A Cell Proliferation and Chromosomal Instability Signature in Anaplastic Thyroid Carcinoma

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Abstract

Here, we show that the anaplastic thyroid carcinoma (ATC) features the up-regulation of a set of genes involved in the control of cell cycle progression and chromosome segregation. This phenotype differentiates ATC from normal tissue and from well-differentiated papillary thyroid carcinoma. Transcriptional promoters of the ATC up-regulated genes are characterized by a modular organization featuring binding sites for E2F and NF-Y transcription factors and cell cycle–dependent element (CDE)/cell cycle gene homology region (CHR) cis-regulatory elements. Two protein kinases involved in cell cycle regulation, namely, Polo-like kinase 1 (PLK1) and T cell tyrosine kinase (TTK), are part of the gene set that is up-regulated in ATC. Adoptive overexpression of p53, p21 (CIP1/WAF1), and E2F4 down-regulated transcription from the PLK1 and TTK promoters in ATC cells, suggesting that these genes might be under the negative control of tumor suppressors of the p53 and pRB families. ATC, but not normal thyroid, cells depended on PLK1 for survival. RNAi-mediated PLK1 knockdown caused cell cycle arrest associated with 4N DNA content and massive mitotic cell death. Thus, thyroid cell anaplastic transformation is accompanied by the overexpression of a cell proliferation/genetic instability-related gene cluster that includes PLK1 kinase, which is a potential molecular target for ATC treatment. [Cancer Res 2007;67(21):10148–58]

Introduction

Anaplastic thyroid carcinoma (ATC) is a highly malignant tumor that accounts for 2% to 5% of all thyroid cancers and is usually seen in the sixth to seventh decades of life. ATC ranks among the most lethal solid tumors, with a median survival of 4 to 12 months after diagnosis (1–3). ATC shares genetic alterations with well-differentiated papillary (PTC) and follicular thyroid carcinomas, namely, point mutations in *RAS* and *BRAF* (1–4) and point mutations or gene amplification of *PIK3CA* (5, 6), suggesting that it may derive from a preexisting differentiated lesion. Whereas well-differentiated thyroid carcinomas are rarely (<10%) *p53*-mutated, more than 70% of ATC are associated with *p53* mutations (2, 4). Besides direct gene mutation, several other mechanisms can obstruct p53 function in thyroid cancer, including the up-regulation of negative p53 regulators like HMGA1 (high-mobility group A1) and ΔNp73, or proteins fostering p53 protein degradation, like HDM2 (7, 8).

ATC has a high proliferation rate and marked aneuploidy (9). Gene expression profiles correlating with mitotic rate and chromosomal instability in cancer cells have been recently defined. A gene expression signature represented by 168 cell cycle–regulated genes has been identified in *in vitro* transformed normal human fibroblasts and termed "the proliferation cluster" (10). Increased expression of many of these genes, in particular of a core set of 44 of them ("44-gene proliferation cluster"), is a surrogate measure of tumor cell proliferation and is often associated with poor outcome (11). Moreover, the up-regulation of a set of 70 genes, known as the chromosomal instability 70 ("CIN70") cluster, partially overlapping with the proliferation signature, predicted aneuploidy in several cancers (12).

Genes coding for two protein kinases, Polo-like kinase 1 (PLK1) and T cell tyrosine kinase (TTK), are included in these signatures (10–12). TTK (also called PYT or hMps1, human monopolar spindle 1 kinase) is a dual-specificity kinase involved in mitotic checkpoint control; it has been implicated in the apoptosis of *p53*-negative cells after DNA damage (13). PLK1 belongs to the *Polo* family of serine/threonine kinases. It is important for many cell cycle–related events, i.e., CDC2 activation, chromosome segregation, centrosome maturation, bipolar spindle formation, activation of the anaphase-promoting complex (APC), and cytokinesis and is overexpressed in many tumors (14). Adoptive overexpression of PLK1 induces NIH 3T3 cell transformation, whereas PLK1 inhibition leads to mitotic arrest and cell death (14).

Here, we show that ATC is characterized by a gene expression profile that overlaps the proliferation and CIN70 signatures, and that PLK1, which is part of these signatures, is essential for survival of ATC cells.

Materials and Methods

Tissue samples. Frozen thyroid tissue samples from 10 patients affected by PTC, 5 patients with ATC, and 4 normal thyroids (N) were used for the microarray screening (Supplementary Table S1). Independent tissue samples (19 normal thyroids, 39 PTC, and 22 ATC) were used for validation assays. All samples were retrieved from the files of the Pathology Department of the University of Pisa (Italy). The study was approved by the Institutional Ethics Committee. The samples were classified according to the diagnostic criteria required for the identification of PTC and ATC (15). The number of neoplastic cells in five areas of 0.2 mm^2 was calculated with a Nikon laser microdissector.

cDNA microarray screening and data analysis. The 12,000 sequence-verified human cDNA set was from the National Genome Institute

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(NIH, Bethesda, MD). The cDNA clones were printed as described elsewhere (16, 17). Sample preparation and microarray hybridizations procedures are reported in detail elsewhere (17). Briefly, total RNA was labeled by direct incorporation of Cy5-dUTP or Cy3-dUTP (Amersham Pharmacia Biotech) in a reverse transcription reaction using anchored oligodeoxythymidylate primer (Genosys) and Superscript II reverse transcriptase (Life Technologies Inc.). Cy3-dUTP–tagged cDNAs were mixed with Cy5-dUTP–tagged common reference and subsequently cohybridized to the microarrays. The reference was composed of a pool of RNA from thyroid cell lines and was used throughout all hybridizations to ensure normalized measures for each gene in each individual sample. Hybridized slides were scanned using an Agilent microarray scanner (Agilent Technologies), and images were processed using a collection of IPLab (Scanalytics, Inc.) extensions developed at the Cancer Genetics Branch, National Human Genome Research Institute. The fluorescence intensities of scanned images were quantified, normalized, and corrected to yield the transcript abundance of a gene as an intensity ratio with respect to that of the signal of the references. Genes were ranked according to the weighted gene analysis. Genes with high weight (w) values create greater separation between groups and denser compaction within the groups, i.e., they have a high power to discriminate between normal thyroid, PTC, and ATC. To test the statistical significance of the discriminative weights, sample labels were randomly permutated as previously described (17). This was repeated 1,000 times to generate a w distribution that would be expected under the assumption of random gene expression, i.e., no difference between the groups. Genes that were deemed to significantly ($P < 0.001$) discriminate between the three categories were listed. The entire data set has been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO)[6] and is accessible through GEO series accession number GSE9115.

RNA extraction and quantitative reverse transcription-PCR. Total RNA was isolated with the RNeasy Kit (Qiagen). About 1 μg of RNA from each sample was reverse-transcribed with the QuantiTect Reverse Transcription (Qiagen). To design a quantitative (Q) reverse transcription-PCR (RT-PCR) assay, we used the Human ProbeLibray system (Exiqon). Primers pairs and PCR conditions are available upon request. PCR reactions were done in triplicate, and fold changes were calculated with the formula: $2^{-(\text{sample 1 } \Delta\text{Ct} - \text{ sample 2 } \Delta\text{Ct})}$, where ΔCt is the difference between the amplification fluorescent thresholds of the mRNA of interest and the mRNA of RNA polymerase 2 used as an internal reference.

Cell cultures. The P5-3N primary culture of normal human thyroid follicular cells was grown as previously described (18). Normal human thyroid cells (S11N and S63N) were grown in RPMI (Invitrogen) containing 20% fetal bovine serum. The human ATC cell lines FB1 (19), BHT101 (20), CAL62 (20), KAT-4 (21), ARO (22), FRO (22), HTH7 (23), HTH83 (23), SW1736 (23, 24), C643 (23), and the poorly differentiated thyroid carcinoma NPA (22) were grown in DMEM (Invitrogen) containing 10% fetal bovine serum. The Fischer rat-derived differentiated thyroid follicular cell line PC Cl 3 (hereafter "PC") was grown in Coon's modified Ham F12 medium supplemented with 5% calf serum and a mixture of six hormones (6H).

Protein studies. Immunoblotting was carried out according to standard procedures. Anti-PLK1 monoclonal antibody was from Zymed Laboratories; anti-TTK polyclonal antibody was from Santa Cruz Biotechnology; monoclonal anti–α-tubulin was from Sigma-Aldrich; anti-cleaved (Asp^{175}) caspase-3 p17 and p12 fragments polyclonal (5A1) was from Cell Signaling Technology, Inc.; anti-poly(ADP-ribose) polymerase (anti-PARP) monoclonal antibody, which detects full-length PARP and the large fragment (89 kDa) produced by caspase cleavage, was from BD Biosciences. Secondary anti-mouse and anti-rabbit antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology.

Reporter gene assay. PCR fragments of the human TTK and PLK1 promoters spanning, respectively, from −524 to +72 and from −148 to +63 relative to the transcription start were cloned into the pGL3 Basic vector (Promega Corporation), carrying the *Firefly* luciferase reporter gene. Primer sequences are available upon request. Cells were transfected using the LipofectAMINE reagent according to manufacturer's instructions, with 500 ng of the reporter plasmid DNA, together with (when required) p53 wild-type, p21(CIP/WAF1) (25), or E2F4 (kindly donated by M. Crescenzi, Istituto Superiore di Sanita', Rome) expression vectors. A plasmid expressing the enzyme *Renilla* luciferase (pRL-*null*) was used as an internal control. In all cases, the total amount of transfected plasmid DNA was normalized by adding empty vector DNA. Forty-eight hours after transfection, *Firefly* and *Renilla* luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega Corporation), and the Lumat LB9507 luminometer (EG Berthold). Each experiment was done in triplicate.

RNA silencing. Small inhibitor duplex RNAs targeting PLK1 and the scrambled control [nonspecific small interfering RNA (siRNA) duplex containing the same nucleotides but in irregular sequence] are described elsewhere (26). Cells were grown under standard conditions. The day before transfection, 5×10^4 cells were plated in 35-mm dishes in DMEM supplemented with 10% fetal bovine serum and without antibiotics. Transfection was done using OligofectAMINE reagent (Invitrogen) with 100 nmol/L (250 nmol/L for PC cells) siRNA. Cells were harvested and counted 24 and 48 h after transfection.

Immunofluorescence. Fixed and permeabilized cells were incubated with anti–α-tubulin antibody (Sigma) for 45 min at 37°C. Washed coverslips were incubated with rhodamine-conjugated secondary antibody (Jackson ImmunoResearch) for 30 min at 37°C. After 15 min propidium iodide (1 μg/mL) counterstaining and coverslips mounting, stained cells were observed with a Zeiss LSM 510 META confocal microscope. The "*In situ* Cell Death Detection Kit, TMR red" (Roche) was used to detect terminal nucleotidyl transferase–mediated nick end labeling (TUNEL)–positive cells. At least 300 cells were counted in triplicate experiments.

Fluorescence-activated cell sorter scan analysis. Cells were harvested and fixed in 70% ethanol for 4 h. After washing with PBS, cells were treated with RNase A (100 units/mL) and stained with propidium iodide (25 μg/mL; Sigma) for 30 min. Samples were analyzed with a CyAn ADP flow cytometer interfaced with the Summit V4.2 software (DakoCytomation). Data were analyzed with the Modfit software (Verity Software House).

Statistical analysis. The two-tailed unpaired Student's t test (normal distributions and equal variances) was used for statistical analysis. All P values were two sided, and differences were significant when $P < 0.05$. All statistical analyses were carried out using the GraphPad InStat software program (version 3.06.3).

Results

Proliferation- and chromosomal instability-associated genes are up-regulated in ATC. We used cDNA microarrays to characterize the gene expression profile of ATC compared with PTC and normal thyroid tissue samples (Supplementary Table S1). Genes were ranked according to weight values (16, 17), with the highest value indicating the most discriminative power ($P < 0.001$) between the different tissue groups. A set of 914 genes distinguished ATC from normal tissue: 371 of them were up-regulated, and 543 were down-regulated in ATC (Supplementary Table S2). Most of these genes were typical of ATC, and only 114 of them discriminated normal thyroid from both PTC and ATC (Supplementary Tables S2 and S3).

ATC featured the up-regulation of a set of 54 genes involved in the control of cell cycle progression and chromosome segregation; 7 and 13 of them were part of the 44-gene proliferation cluster (10, 11) and the CIN70 cluster (12), respectively, and 9 of both clusters (Table 1). These genes were not significantly up-regulated in PTC (Supplementary Table S3). Additional genes of the two clusters tended toward up-regulation in ATC, but the data were not statistically significant (Supplementary Table S4). Thus, ATC overexpressed genes that coded for cyclins (*CCNB2,*

[6] http://www.ncbi.nlm.nih.gov/geo

Table 1. Proliferation- and chromosomal instability-related genes up-regulated in ATC

Gene	Gene description (gene ontology biological process)	Unigene number	Weight N versus ATC
ZWINT	ZW10 interactor (cell cyle)	Hs.42650	31.36
KNSL6	KIF2C kinesin family member 2C (mitosis)	Hs.69360	29.69
CENPA¶	Centromere protein A (17 kDa; mitosis)	Hs.1594	27.61
NEK2¶	NIMA (never in mitosis gene a)-related kinase 2 (cell cycle)	Hs.153704	21.77
CEP55	Centrosomal protein (55 kDa; cell cycle)	Hs.14559	19.15
CDC20¶	Cell division cycle 20 homologue (cell cycle)	Hs.82906	17.71
CENPF	Centromere protein F (350/400 kDa, mitosin; mitosis)	Hs.77204	15.24
PARP1	Poly(ADP-ribose) polymerase family 1 (adenosine diphosphate ribosyltransferase; base excision repair)	Hs.177766	14.60
SSRP1	Structure-specific recognition protein 1 (DNA replication)	Hs.523680	14.39
CCNB2¶	Cyclin B2 (cell cycle)	Hs.194698	14.01
PBK	PDZ binding kinase (mitosis)	Hs.104741	13.98
TTK¶	TTK protein kinase (mitotic spindle checkpoint)	Hs.169840	13.52
EXOSC9	Exosome component 9 (rRNA processing)	Hs.91728	13.18
CDCA8¶	Cell division cycle–associated 8 (cell cycle)	Hs.48855	12.93
CCNA2	Cyclin A2 (cell cycle)	Hs.58974	12.14
TOP2A¶	Topoisomerase (DNA) IIα (DNA topological change)	Hs.156346	11.87
PLK1¶	Polo-like kinase 1 (mitosis)	Hs.329989	11,23
GMPS	Guanine monophosphate synthetase (purine synthesis)	Hs.518345	11.17
PPM1G	Protein phosphatase 1G (cell cycle)	Hs.17883	10.81
CENPE	Centromere protein E (mitotic metaphase)	Hs.75573	10.79
PRC1¶	Protein regulator of cytokinesis 1 (mitotic spindle elongation)	Hs.366401	10.71
NOL5A	Nucleolar protein 5 (rRNA processing)	Hs.376064	10.22
TPX2	Microtubule-associated, homologue (mitosis)	Hs.244580	9.92
NUSAP1	Nucleolar and spindle-associated protein 1 (mitosis)	Hs.615092	9.82
ZWILCH	Kinetochore-associated, homologue (kinetochore component)	Hs.21331	9.47
NCAPH	Non-SMC condensin I complex, subunit H (mitosis)	Hs.308045	9.39
HMMR	Hyaluronan-mediated motility receptor (cell motility)	Hs.72550	9.31
PAICS	Phosphoribosylaminoimidazole carboxylase (purine synthesis)	Hs.331420	9.30
RCC1	RCC1 regulator of chromosome condensation 1 (cell cycle)	Hs.469723	8.93
CTPS	CTP synthase (nucleotide metabolism)	Hs.473087	8.58
TYMS	Thymidylate synthetase (nucleotide metabolism)	Hs.592338	7.96
CKS1	CDC28 protein kinase regulatory subunit 1B (cell cycle)	Hs.374378	7.93
FOXM1	Forkhead box M1 (regulation of transcription)	Hs.239	7.91
HNRPF	Heterogeneous nuclear ribonucleoprotein F (RNA processing)	Hs.808	7.53
HPRT1	Hypoxanthine phosphoribosyltransferase 1 (purine synthesis)	Hs.412707	7.08
SMC4	Structural maintenance of chromosomes 4 (cell cycle)	Hs.58992	6.83
CCNE2	Cyclin E2 (cell cycle)	Hs.567387	6.79
GMNN	Geminin, DNA replication inhibitor (cell cycle)	Hs.234896	6.66
UBE2S	Ubiquitin-conjugating enzyme E2S (ubiquitin cycle)	Hs.396393	6.56
CDC2	Cell division cycle 2 (mitosis)	Hs.334562	6.55
GINS2	GINS complex subunit 2 (DNA replication)	Hs.433180	6.30
PLK4	Polo-like kinase 4 (cell cycle)	Hs.172052	6.28
DNMT1	DNA (cytosine-5-)-methyltransferase 1 (DNA methylation)	Hs.202672	5.54

(*Continued on the following page*)

Table 1. Proliferation- and chromosomal instability-related genes up-regulated in ATC (Cont'd)

Gene	Included in			p53 inhibited	DNA elements (binding sites)			
	Proliferation cluster*	44-gene proliferation cluster †	CIN70 ‡		E2F	NF-Y	CDE	CHR
ZWINT	Yes		Yes	Yes§,‖	Yes*	Yes*	Yes*	
KNSL6	Yes			Yes§				
CENPA¶	Yes			Yes§,‖		Yes*	Yes*	Yes*
NEK2¶	Yes		Yes	Yes‖,**				
CEP55			Yes					
CDC20¶	Yes	Yes	Yes	Yes§,‖,**	Yes*	Yes*		
CENPF	Yes	Yes		Yes§,‖	Yes*	Yes*,† †	Yes*	Yes*,† †
PARP1	Yes							
SSRP1	Yes							
CCNB2¶	Yes		Yes	Yes§,**	Yes*	Yes*,† †		Yes*,† †
PBK	Yes		Yes	Yes‖				
TTK¶	Yes		Yes	Yes§	Yes † †	Yes*,† †		Yes*,† †
EXOSC9	Yes	Yes						
CDCA8¶			Yes		Yes*	Yes*,† †	Yes	Yes*,† †
CCNA2	Yes	Yes		Yes§,‖	Yes*		Yes*	
TOP2A¶	Yes	Yes	Yes	Yes§,‖,**		Yes*,† †		Yes*,† †
PLK1¶	Yes	Yes		Yes§,**	Yes ‡ ‡	Yes*,† †	Yes*	Yes*,† †
GMPS	Yes			Yes§		Yes*	Yes*	
PPM1G	Yes							
CENPE		Yes						
PRC1¶	Yes		Yes	Yes‖	Yes*	Yes*,† †	Yes*	Yes*,† †
NOL5A	Yes					Yes*	Yes*	
TPX2			Yes	Yes§,‖	Yes*	Yes*		
NUSAP1	Yes			Yes‖	Yes*	Yes*,† †	Yes*	Yes*,† †
ZWILCH			Yes					
NCAPH			Yes					
HMMR	Yes			Yes§,‖,**		Yes*,† †		Yes*,† †
PAICS	Yes			Yes‖				
RCC1	Yes							
CTPS		Yes	Yes					
TYMS		Yes			Yes*		Yes*	
CKS1	Yes				Yes*	Yes*	Yes*	Yes*
FOXM1			Yes	Yes§,‖		Yes*,† †		Yes*,† †
HNRPF	Yes			Yes**	Yes*	Yes*	Yes*	
HPRT1	Yes							
SMC4	Yes			Yes‖				
CCNE2	Yes			Yes‖				
GMNN	Yes				Yes*		Yes*	
UBE2S	Yes			Yes§,**				
CDC2	Yes		Yes	Yes‖	Yes*,† †	Yes*,† †	Yes*	Yes*,† †
GINS2	Yes			Yes§				
PLK4	Yes							
DNMT1		Yes						

(*Continued on the following page*)

Table 1. Proliferation- and chromosomal instability-related genes up-regulated in ATC (Cont'd)

Gene	Gene description (gene ontology biological process)	Unigene number	Weight N versus ATC
FEN1	Flap structure-specific endonuclease 1 (DNA replication)	Hs.409065	5.38
C20orf24	Chromosome 20 open reading frame 24 (RAB5-interacting protein)	Hs.584985	5.34
MCM5	Minichromosome maintenance-deficient 5 (DNA replication)	Hs.517582	5.32
UBE2C	Ubiquitin-conjugating enzyme E2C (mitosis)	Hs.93002	5.09
MCM7	Minichromosome maintenance-deficient 7 (DNA replication)	Hs.438720	5.00
ATAD2	ATPase family, AAA domain containing 2 (ATP binding)	Hs.370834	4.69
POLE	Polymerase (DNA directed), epsilon (DNA replication)	Hs.524871	4.30
CKS2	CDC28 protein kinase 2 (cell cycle)	Hs.83758	4.25
MCM6	Minichromosome maintenance-deficient 6 (DNA replication)	Hs.444118	4.25
CDC25C	Cell division cycle 25C (cell cycle)	Hs.656	4.05
RFC1	Replication factor C (activator 1) 1 (DNA replication)	Hs.507475	4.05

*Ref. 10.
†Ref. 11.
‡Ref. 12.
§Ref. 13.
‖Ref. 33.
¶Genes whose up-regulation was studied also by Q-RT-PCR.
**Ref. 32.
† † Ref. 28.
‡ ‡ Ref. 38.

CCNA2, and *CCNE2*), cyclin-dependent kinases (*CDC2*), and proteins involved in nucleotide synthesis (*GMPS, PAICS, CTPS, TYMS*, and *HPRT1*), spindle formation, and checkpoint control (*PLK1, TTK, NEK2, CDCA8, CENPA, CENPF, CENPE*, and *KNSL6*; Table 1). According to recent reports, ATC overexpressed *UBE2C* (*UBCH10*), which encodes an E2 ubiquitin-conjugating enzyme that is required for cell cycle progression (27), and genes of the minichromosome maintenance–deficient (*MCM*) family, involved in licensing DNA for replication (25). Genes encoding other components of the DNA replication initiation complex, such as *CDC6, ORC1*, and *PCNA*, also tended toward up-regulation in ATC (Supplementary Table S4). ATC frequently features genetic alterations in the phosphoinositide-3-kinase (PI3K)/AKT pathway (5, 6); accordingly, the screening revealed the altered expression of some genes of this pathway in ATC samples (Supplementary Table S5).

Some cis-regulatory DNA elements, e.g., binding sites for E2F and NF-Y (CCAAT box binding) transcription factors and cell cycle-dependent element (CDE)/cell cycle gene homology region (CHR) DNA orphan binding sites, were highly represented in the promoters of the 54 genes up-regulated in ATC (refs. 10, 28–30; Table 1). The E2F family of transcription factors includes positive (E2F1-3) and negative (E2F4-6) regulators of cell cycle progression (28, 29). Activator E2Fs (in particular E2F1) are linked to the activation of genes involved in G_1-S progression, whereas repressor E2Fs (in particular E2F4) bind gene promoters featuring the NF-Y-CDE/CHR DNA module that often peak at the G_2 or G_2-M phase (10, 28–30). Many G_2-M phase genes (like *PLK1, TTK, PRC1, CENPE, CENPF, FOXM1, CCNB2, CDC2, CDCA8, CCNA2, TOP2A, UBE2C*, and *CDC25C*) were up-regulated in ATC. Based on such a promoter architecture, it can be argued that some of the gene expression changes associated to ATC may be influenced by the loss of *p53* or *pRB* family gene function, which is able to influence the function of E2F and NF-Y proteins (10, 30–33). Accordingly, as many as 31 of the 54 genes up-regulated in ATC samples were previously reported to be under p53 negative control (Table 1; refs. 13, 32, 33). Although the *p53* gene status of the examined ATC samples is unknown, the microarray screening (Supplementary Table S2) showed that four out of five ATC samples overexpressed HMGA1 (weight = 4.3), a negative regulator of the p53 function (7).

We selected an independent set of ATC samples for validation experiments. A total of 2 out of 10 of these samples harbored R248E and R280T *p53* mutations, respectively (data not shown). Moreover, five of them up-regulated HMGA1 mRNA at a RT-PCR analysis (Supplementary Fig. S1). These samples were examined by triplicate quantitative RT-PCR for the expression of 10 of the genes reported in Table 1. The results of these experiments were in agreement with the microarray screening data. Although the expression level of single genes varied among individual ATC samples, these genes were up-regulated in practically all ATC samples examined versus normal thyroids and PTC (Fig. 1*A*). The difference between ATC and normal tissue was $P < 0.001$ with the Tukey-Kramer multiple comparisons procedure and $P < 0.0001$ with the one-way ANOVA test. We also measured the expression levels of the 10 genes in a panel of ATC cell lines expressing a mutated *p53* allele (34) in comparison to normal thyroid cells. All of them invariably up-regulated in cancer cells (Table 2).

Table 1. Proliferation- and chromosomal instability-related genes up-regulated in ATC (Cont'd)

Gene	Included in			p53 inhibited	DNA elements (binding sites)			
	Proliferation cluster*	44-gene proliferation cluster †	CIN70 ‡		E2F	NF-Y	CDE	CHR
FEN1	Yes	Yes	Yes	Yes§,‖				
C20orf24			Yes		Yes*	Yes*	Yes*	
MCM5	Yes	Yes			Yes*,† †	Yes*		
UBE2C	Yes		Yes	Yes§,‖		Yes*,† †		Yes*,† †
MCM7			Yes	Yes§,‖,**	Yes*	Yes*		
ATAD2			Yes					
POLE	Yes			Yes‖	Yes*	Yes*	Yes*	
CKS2	Yes	Yes	Yes	Yes§		Yes*,† †	Yes*	Yes*,† †
MCM6	Yes	Yes		Yes‖,**	Yes*,† †	Yes*	Yes*	
CDC25C	Yes	Yes			Yes*	Yes*		
RFC1		Yes			Yes*		Yes*	

PLK1 and TTK transcriptional promoters are negatively controlled by p53 in ATC cells. We focused on *PLK1* and *TTK*, which encode two protein kinases involved in cell cycle progression (13, 14). As shown in Table 2, the ATC cell lines analyzed overexpressed PLK1 and TTK compared with the average expression level of normal thyrocytes ($P < 0.0001$). NPA cells, which are derived from a poorly differentiated thyroid carcinoma, also up-regulated PLK1 and TTK mRNAs. The up-regulation of PLK1 and TTK occurred also at protein level (Fig. 1*B*).

We cloned the PLK1 gene transcriptional promoter (−148 to +63; ref. 35) and a putative TTK promoter (−524 to +72; ref. 28) in the pGL3 vector upstream from the *Firefly* luciferase reporter (Fig. 2*A*). We transiently transfected the pGL3-PLK1-LUC and pGL3-TTK-LUC constructs (or the empty vector) in triplicate in a continuous line of normal thyrocytes (PC) and in ATC cells and measured luciferase activity. Transcription from the TTK ($P < 0.0013$) and the PLK1 ($P < 0.0019$) promoters was strongly up-regulated in ATC with respect to normal cells (Fig. 2*B*).

TTK and PLK1 feature the typical combination of E2F, NF-Y, and CHR elements (Table 1) and were reported to be negatively controlled by p53 (13, 32). To explore the mechanism of PLK1 and TTK up-regulation in ATC cells, we measured the effects of wild-type p53 and the cyclin-dependent kinase inhibitor p21 (CIP1/WAF1). As shown in Fig. 2*C*, adoptive overexpression of both p53 and p21 decreased TTK (~2- and 3-fold, respectively) and PLK1 (~2–3- and 3–5-fold, respectively) promoter activity in FRO and CAL62 cells ($P < 0.0001$). Transient expression of the negative E2F, E2F4, also reduced the activity of both promoters in ATC cells ($P < 0.0001$; Fig. 2*C*).

PLK1 knockdown induces mitotic death of ATC cells. PLK1 depletion or inactivation decreased the viability of several tumor cell types (14, 26, 36, 37). We used the RNA interference method to deplete endogenous PLK1 from ATC cells; normal PC thyrocytes served as a control. Twenty-four hours after transfection with PLK1-specific siRNA, PLK1 protein was silenced in CAL62 (≥75%) and ARO (~60%) cells, whereas a scrambled control had no effect (Fig. 3*A* and *B*). Forty-eight hours post-transfection, CAL62 cells treated with scrambled RNAi numbered 381×10^3, whereas those treated with PLK1 RNAi numbered 73×10^3 ($P < 0.0001$; Fig. 3*A*); ARO cells treated with scrambled RNAi numbered 285×10^3, and those treated with PLK1 RNAi numbered 106×10^3 ($P = 0.0014$; Fig. 3*B*). Virtually no effect was observed in normal cells, although RNAi depleted PLK1 in PC cells, albeit with a lower efficiency than in cancer cells (Fig. 3*C*).

To better characterize the effects of PLK1 knockdown, we transfected CAL62 cells with PLK1 siRNA or the scrambled control and analyzed them by immunoblot at different time points. Thirty-six hours post-transfection, two biochemical markers of apoptosis, i.e., cleaved products of caspase-3 and of PARP, were visible in PLK1-silenced cells but not in cells transfected with the scrambled control (Fig. 4*A*). Accordingly, PLK1 depletion, but not control RNAi, strongly increased the rate of inter-nucleosomal DNA fragmentation in a TUNEL assay (Fig. 4*B*). Forty-eight hours after transfection, the percentage of apoptotic cells with a subgenomic DNA (sub-G_1) content at the fluorescence-activated cell sorter (FACS) was higher in PLK1-depleted cells than in control cells (14% versus 4%; data not shown). In addition, the fraction of cells with a 4N DNA complement was higher in PLK1-depleted cells (59% versus 14%) versus control cells, which indicates incomplete cytokinesis (data not shown).

We stained PLK1-silenced and control-treated CAL62 cells with anti–α-tubulin antibody and examined the mitotic spindle by confocal microscopy. Consistent with the FACS results, the number of mitotic (prometaphase-like state) cells was increased in PLK1-depleted cells as early as 12 h post-transfection. Forty-eight hours after transfection, 53 ± 5% of PLK1-depleted cells versus 3 ± 2% of control-treated cells were in the M phase (Fig. 4*C*). As shown in Fig. 4*D*, PLK1-depleted cells had monopolar (20% versus 0% in control cells) and disorganized (48% versus 9.8% in control cells) spindles. Moreover, in PLK1-depleted cells, there was an increase of bi-nucleated cells (5.4% versus 1.4% in control cells) and cells with an aberrant nuclear morphology (dumbbell-like chromatin; 12% versus 4.3% in control cells; data not shown). A dumbbell-like structure suggests the inability to separate sister chromatids at the onset of anaphase. This feature is a hallmark of so-called mitotic cell death or mitotic catastrophe, a type of apoptosis cells that are committed to when they are unable to complete cytokinesis.

Discussion

This study shows that ATC is characterized by the overexpression of genes associated with cell proliferation and chromosomal instability. This feature, not being detected in well-differentiated PTC, is typical of ATC and is consistent with the highly mitogenic and aneuploid ATC phenotype (1, 2, 9). The promoters of most of the proliferation-related genes up-regulated in ATC contain a typical combination of NF-Y and E2F binding sites and CDE/CHR cis-elements, a feature that suggests an indirect control exerted by p53 and pRB tumor suppressors. Consistently, we could show that TTK and PLK1 promoters are negatively controlled by p53-p21(CIP/WAF1) axis in ATC cells.

Activator E2Fs-regulated promoters are repressed by pRB family members; such a repression is alleviated on by pRB phosphorylation by cyclin-associated kinases (CDK; refs. 29, 38). By stimulating increased levels of the CDK inhibitor p21(CIP/WAF1), p53 reduces pRB phosphorylation levels and, in turn, E2F transcriptional activity (29). Moreover, CDK2 activity, and therefore, p53-mediated p21(CIP/WAF1) induction, also controls NF-Y through direct phosphorylation (28). Finally, by directly associating with NF-Y, p53 suppresses NF-Y binding promoters, like the *CCNB2, CDC25C*, and *CDC2* gene promoters (31).

The CDE/CHR DNA elements are targets of repressor E2Fs (E2F4 and 5; ref. 30). E2F4 is under the negative control of the pRB-like

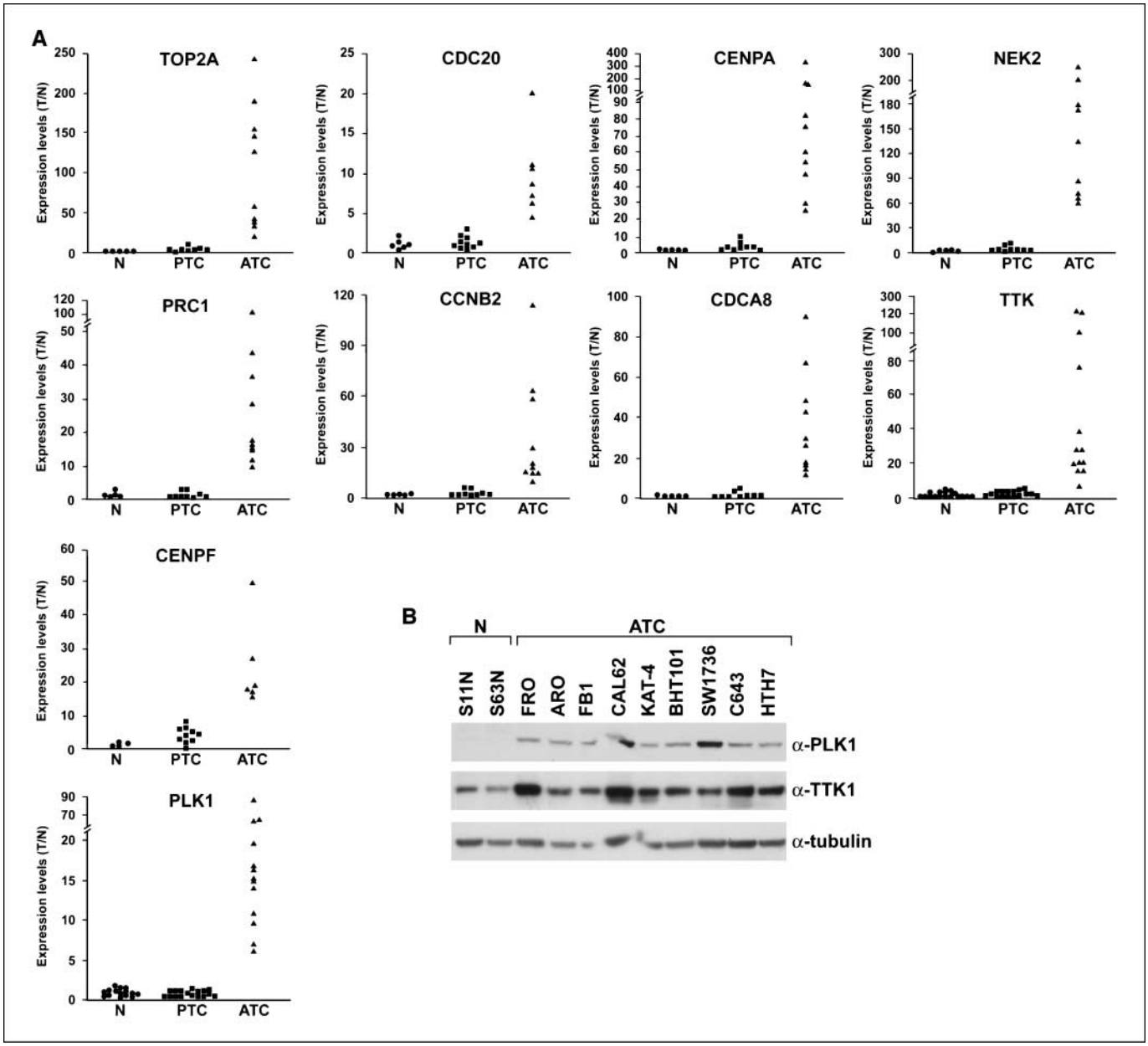

Figure 1. *A,* expression levels of ATC-specific genes in human thyroid samples. Quantitative RT-PCR analysis of 10 ATC-specific genes in normal thyroid (*N*), PTC, and ATC samples. TTK and PLK1 expression was measured in 20 N, 16 PTC, and 13 ATC samples. The other eight genes were measured in five N, nine PTC, and six ATC samples. For each gene, the expression level in a given tumor sample (*T*) was obtained by comparing its fluorescence threshold with the average fluorescence thresholds of normal samples (*N*). Values are the average results of three independent determinations. *B,* PLK1 and TTK protein levels in human thyroid cell lines. Fifty micrograms of protein lysate harvested from primary cultures of normal human thyroid follicular (*N*) and ATC cells were examined by immunoblot using anti-PLK1 and anti-TTK antibodies. Equal protein loading was verified with an anti–α-tubulin antibody. Data are representative of three independent experiments.

Table 2. Expression levels of ATC-specific genes in human ATC cell lines

Expression level (fold change ATC cells versus normal thyrocytes)*

Cell line	P53 status	TOP2A	CENPA	PRC1	CCNB2	CDCA8	NEK2	TTK	PLK1	CDC20	CENPF
ARO	R273H [†,‡]	1.6	2.4	6.1	3.9	4.3	6.8	8	6.5	2	4
SW1736	Null [§]	8.8	13.5	7.7	10.4	12.9	18	25	81	ND	ND
NPA	G266V [†,‡]	4.8	14.4	8.1	9	6.3	11.7	16.5	46.6	8	8
BHT101	I251T [‡]	2.1	3.5	1.8	2.2	2.5	3.6	16.7	10.8	ND	ND
KAT-4	R273H [†,‡]	6.7	5.9	5.5	9.7	9.2	14	13	27.1	10	8
C643	R248Q [‡]	2.7	3.9	2.5	4	6.5	5.9	25.5	8.3	ND	ND
CAL62	A161D [‡]	ND	ND	ND	ND	ND	ND	25.7	25.7	8	8
FRO	Null [†]	3.8	3.9	3.1	3.5	4.5	9.1	32.5	14.3	ND	ND

NOTE: Values are the average results of three independent determinations.
Abbreviation: ND, not done.
*Quantitative RT-PCR analysis done as indicated in the legend to Fig. 1*A*. For each gene, the expression level in a given ATC cell line was obtained by comparing its fluorescence threshold with the average fluorescence thresholds of two normal thyroid cell populations (P53N and S11N).
†Ref. 22.
‡Ref. 34.
§Ref. 24.

p107 (RBL1) and p130 (RB2) proteins, and thus, also E2F4 and pRB-like proteins might be implicated in the regulation of proliferation-associated genes in ATC. We could indeed show that TTK and PLK1 promoters are negatively controlled by E2F4 in ATC cells.

Finally, gene promoters containing NF-Y/CDE-CHR modules often contain ELK1-binding sites (10, 28). ELK1 transcription factors are downstream targets of the mitogen-activated protein kinase pathway, and ATC often features mutation in genes (RAS or

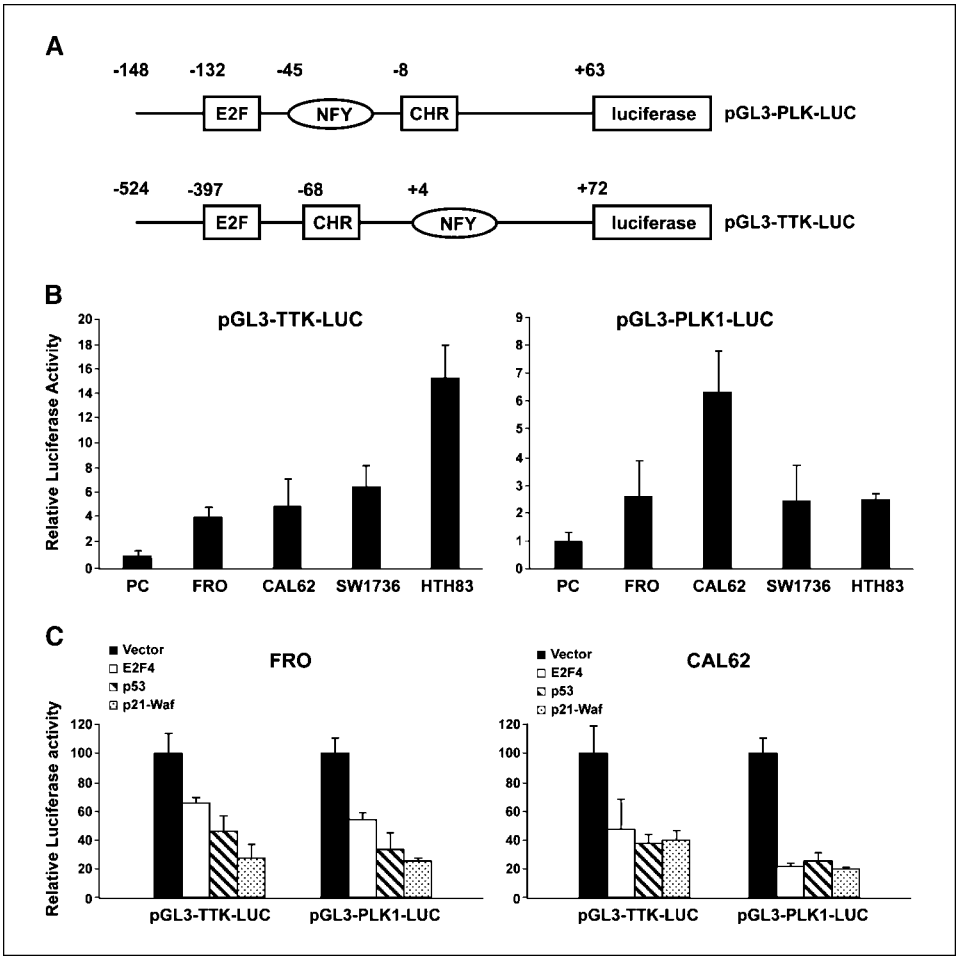

Figure 2. PLK1 and TTK gene promoter activity in ATC cells. *A,* the two promoters are schematically represented. *B,* the indicated cell lines were transiently transfected with pGL3-TTK-LUC (*left*) or pGL3-PLK1-LUC (*right*) plasmids. The average levels of luciferase activity in three independent experiments are reported. *Bars,* 95% confidence intervals. *C,* FRO and CAL62 cells were co-transfected with p53, p21(CIP/WAF1), or E2F4 expression vectors (or the empty vector), together with pGL3-TTK-LUC (*left*) or pGL3-PLK1-LUC (*right*). Relative luciferase activity is reported. The average results of three independent assays are reported, and 95% confidence intervals are shown; promoter activity values in vector-transfected cells were arbitrarily set at 100.

BRAF) in this cascade (1–4). Therefore, it is likely that pathways other than the p53 and pRB ones concurrently regulate the expression of genes of the proliferation cluster.

ATC surgery is often only palliative, and there is no effective systemic treatment (2). PLK1 kinase inhibitors are currently being evaluated in clinical trials for various cancer types (14, 26, 36, 37). Here, we show that PLK1 is required for ATC cell proliferation and survival. This requirement was restricted to ATC cells and was not detected in normal thyroid follicular cells. Although the exact mechanism of this selectivity is unknown, it has been shown that p53 depletion increases the sensitivity of various cell types to PLK1 knockdown (36, 37). Whatever the mechanism, our findings indicate

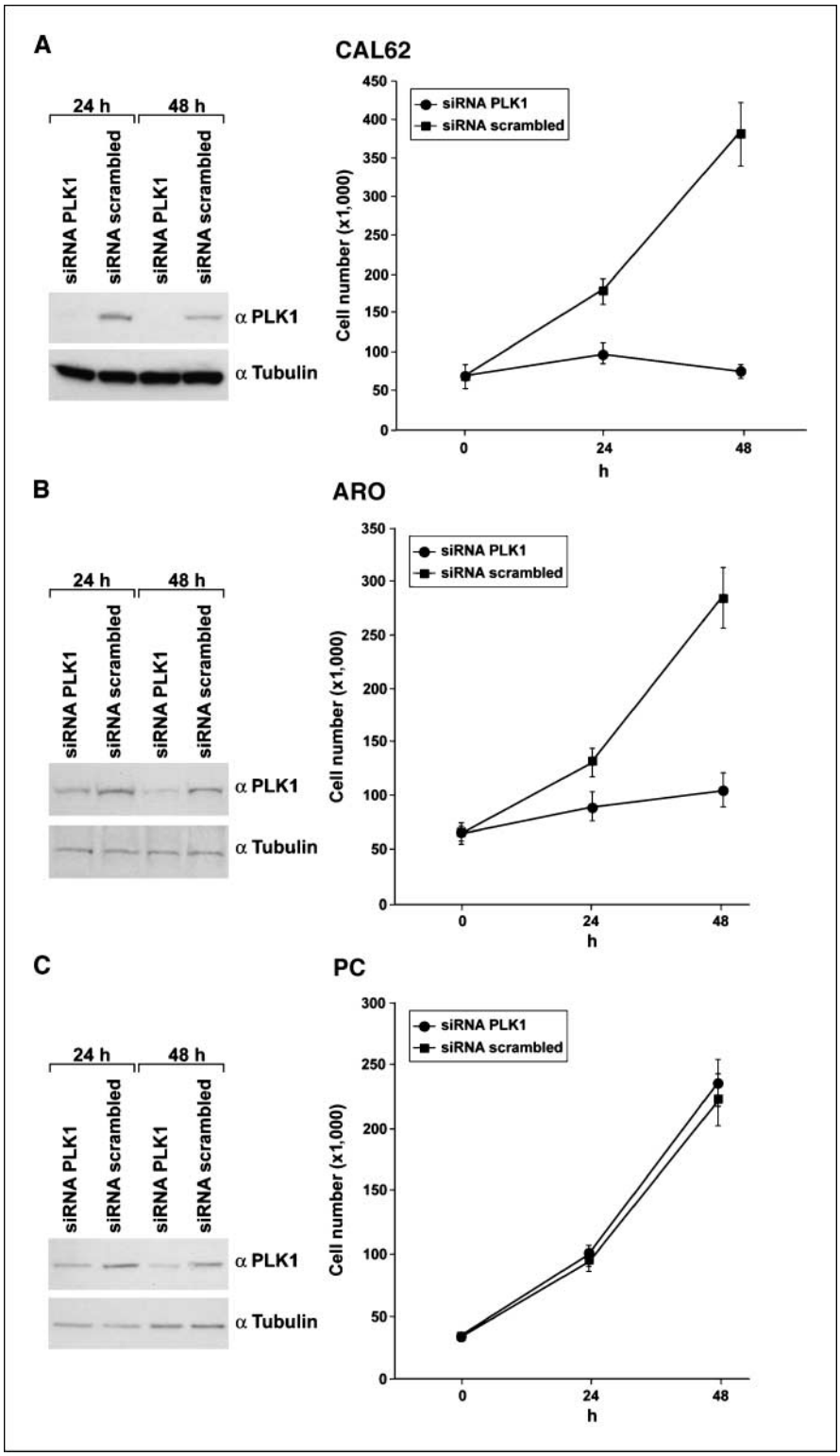

Figure 3. Effects of PLK1 knockdown on ATC cell growth. CAL62 (*A*), ARO (*B*), and normal PC (*C*) cells were transfected with either PLK1-siRNA or the scrambled oligonucleotide control. Cells were harvested and counted at the indicated time points. Values represent the average of triplicate determinations. *Bars,* 95% confidence intervals (*right*). Equal amounts of protein lysates were subjected to immunoblot with anti-PLK1 or α-tubulin antibodies (*left*).

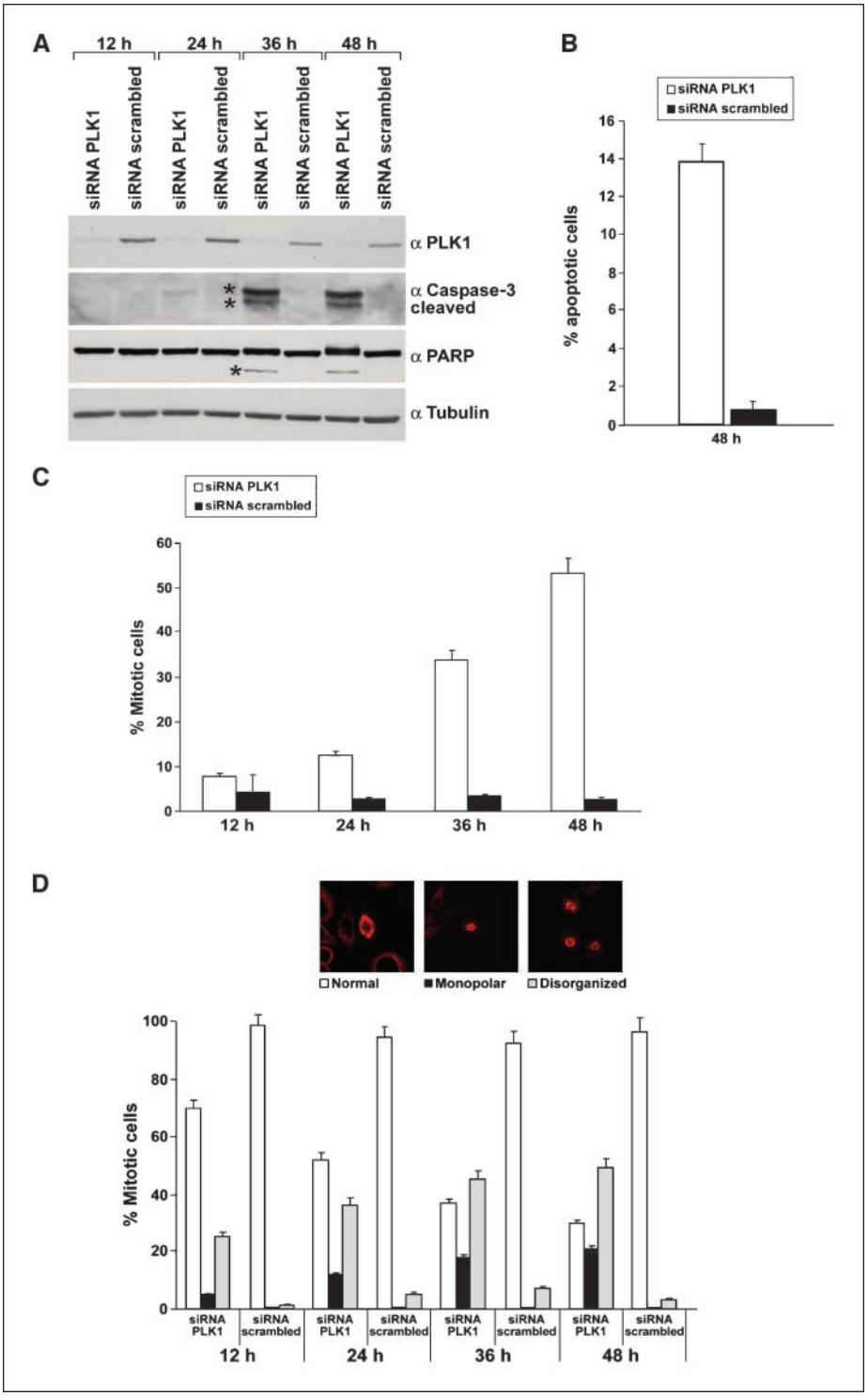

Figure 4. Effects of PLK1 knockdown on apoptosis and cell cycle progression in ATC cells. *A,* CAL62 cells transfected with either PLK1 siRNA or its scrambled control were lysed at the indicated time points and subjected to immunoblot with anti-cleaved caspase-3 antibodies, which detect only the p17 and p12 cleaved caspase-3 fragments (*) and anti-PARP antibodies, which detect both the intact 116 kDa and its cleaved 89-kDa (*) fragment. Anti-PLK1 immunoblot was used to verify RNAi; α-tubulin levels are shown for normalization. Data are representative of at least three different experiments. *B,* TUNEL analysis of PLK1-siRNA and scrambled control-treated CAL62 cells (48 h posttransfection). The percentage of positive cells represents the average value of triplicate experiments ± SD, in which at least 300 cells were counted. *C,* CAL62 cells, grown on glass coverslips, were transfected with PLK1-siRNA or with its scrambled control sequence. At the indicated time points, cells were fixed, stained with anti–α-tubulin, and analyzed by confocal microscopy. The percentage of mitotic cells represents the average ± SD of three experiments in which at least 400 cells were counted. *D,* mitotic cells with normal spindles, monopolar spindles, or grossly disorganized spindles were counted. Values refer to the average ± SD of triplicate experiments in which at least 200 mitotic cells were counted; *insets,* representative images.

that PLK1 addiction might be an Achilles' heel of ATC cells and might be exploited to develop novel treatment strategies for this cancer.

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